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24 h Excretion Patterns of Free, Conjugated and Methylated Catecholamines in Man

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Summary: A method based on high performance liquid chromatography with electrochemical detection is described for quantification of adrenaline, noradrenaline, dopamine (free and total), metanephrine, normetanephrine, and 3-methoxytyramine (free) in urine samples. Purification of the samples is achieved on disposable Bio-Rad ion-exchange columns in combination with small Sephadex G 10 columns. A clear circadian rhythm was found in the 24 h excretion pattern of free adrenaline and noradrenaline for 4 volunteers (city bus drivers during a working day). No rhythms were seen in the 24 h excretion pattern of free or conjugated dopamine, conjugated adrenaline or conjugated noradrenaline, or of the methylated free catecholamines. It is concluded that in order to obtain information about the neurotransmitter function of peripheral catecholamines it is sufficient to determine the non-conjugated compounds; values for the conjugated and methylated metabolites contribute no extra information.

24-Stunden-Ausscheidungsmuster der freien, konjugierten und methylierten Katecholamine beim Menschen

Zusammenfassung: Eine Methode zur quantitativen Bestimmung von Adrenalin, Noradrenalin, Dopamin (freies und gesamtes), Metanephrin, Normetanephrin und 3-Methoxytyramin (freies) im Harn auf der Grundlage der Hochleistungsflüssigchromatographie mit elektrochemischer Detektion wird beschrieben. Die Reinigung der Proben wird durch Einmal-Ionenaustauscher-Säulen (Bio-Rad) in Kombination mit kleinen Sephadex G 10-Säulen erreicht. Bei vier Busfahrern wurde während eines Arbeitstages ein deutlicher circadianer Rhythmus im 24-Stunden-Ausscheidungsmuster für freies Adrenalin und Noradrenalin gefunden. Für freies oder konjugiertes Dopamin, konjugiertes Adrenalin, konjugiertes Noradrenalin oder die methylierten freien Katecholamine wurde keine Rhythmik im 24-Stunden-Ausscheidungsmuster beobachtet. Wir schließen daraus, daß es für den Erhalt von Informationen über die Neurotransmitterfunktion der peripheren Katecholamine ausreicht, die nicht-konjugierten Verbindungen zu bestimmen; Werte für die konjugierten und methylierten Metabolite erbringen keine weitere Information.

Introduction

Psychoendocrine research has emphasized the use of catecholamines as indicators of mental stress and physical workload (1, 2). Adrenaline (A) excretion appears to be particulary responsive to mental stress, whereas urinary noradrenaline responds mainly to physical activity (3, 4). Although studies in this field are usually restricted to the free catecholamines, there is some recent evidence that also methylated catecholamines should be taken into account as indices of sympathicoadrenal activity (5). It is believed that O-methylation of catecholamines by the extraneuronally localized enzyme, catechol-O-methyl transferase, is the primary metabolic route for actively released catecholamine. This enzyme converts noradrenaline, adrenaline and dopamine into normetanephrine, metanephrine, and 3-methoxytyramine, respectively.

Several processes, based on photometry (6), fluorimetry (7), HPLC (8) and mass spectroscopy (9) have been developed for analysis of total urinary normetanephrine, metanephrine and 3-methoxytyramine. However, these methods were usually developed for diagnosis of catecholamine secreting tumours, as is the case in pheochromocytoma. In contrast to the total (free plus conjugated) urinary metanephrines, data on the levels of the free metanephrines are relatively scarce in the literature (10, 11).

Here, we describe a new method for simultaneous analysis of catecholamines (free and total) as well as for free normetanephrine, metanephrine and 3-methoxytyramine in human urine. The method is an extension of our earlier work in which we described the analysis of free catecholamines in urine (10). The assay is based on a two step isolation procedure, first by adsorption on a prepacked ion exchange column (Bio Rad Catecholamine Kit) followed by purification on a small Sephadex G 10 column. The purified samples were quantified by HPLC in conjunction with an electrochemical detector.

Complete 24 h excretion patterns of adrenaline, noradrenaline, dopamine (free as well as conjugated), normetanephrine, metanephrine and 3-methoxytyramine (only free) were determined for 4 volunteers. The significance of the determination of catecholamines and related metabolites in urine for psychoendocrine research is discussed.

Materials and Methods

Standards and reagents

Materials and their sources were as follows: noradrenaline bitartrate, adrenaline bitartrate, normetanephrine · HCl, metanephrine · HCl, 3-methoxytyramine · HCl (Sigma Chemical Co., St. Louis, MO 63176), dopamine · HCl, dihydroxybenzylamine · HBr (DHBA, Janssen Pharmaceutica, Beerse, Belgium), ion-exchange columns (Catecholamine Kit, disposable columns filled with Bio-Rex 70, Bio-Rad laboratories GmbH, D-8000, München, F. R. G.), 1-heptanesulphonic acid disodium salt (Kodak, Rochester N. Y., U. S. A.), Sephadex G 10 (Pharmacia, Uppsala, Schweden). All other chemicals were of analytical reagent grade and were purchased from E. Merck (Darmstadt, F. R. G.). All aqueous solutions of noradrenaline, adrenaline, dopamine, normetanephrine, metanephrine and 3-methoxytyramine contained 100 mg/l in 30 mmol/l hydrochloric acid and were stored at −20 °C. Every two weeks, standard solutions were freshly prepared from a portion of the stock solution by appropriate dilution with 30 mmol/l hydrochloric acid.

Urine collection

Four city bus drivers (subjects 1, 2, 3, and 4), 30−40 years of age, volunteered in this investigation. The drivers were selected from a population of 28 bus drivers who participated in an investigation of the effect of workload on neuroendocrine responses. It appeared from this study that the adrenaline excretion was strongly increased in response to workload (12). All subjects were in good health at the time according to medical examination just before the experiment. No dietary restrictions were placed upon the drivers. Subject 1 was included as this bus driver was known to excrete high amounts of adrenaline.

Urine samples were collected over a complete 24 h period during a normal working day. Eight samples were collected: at 7.00 (night sample), 9.00, 12.30, 14.00, 17.00, 18.00, 20.00, and 22.0 h. Samples were preserved with sodium metabisulphite (0.5 g). On completion of collection, the pH was adjusted to between 3 and 4 with concentrated formic acid. After measuring the volume of the specimen, we removed about 20 ml for analysis. The aliquot, stored at −80 °C, was stable for longer than six months.

Creatinine was determined with an Autoanalyzer method (Technicon, SMA 6).

Purification of urine samples

Isolation of catecholamines and methylated metabolites on prepacked Bio-Rad ion-exchange columns (filled with Bio-Rex 70) was performed as indicated by the manufacturer, with some minor modifications: centrifuged urine (5 ml) was mixed with 2.7 mmol/l Na_2-EDTA (15 ml) and adjusted to pH 6.5 ± 0.1 with 0.5 mol/l sodium hydroxide. One fifth of this mixture was applied to the ion-exchange column, which was then washed twice with 8 ml of water. Free catecholamines were eluted with 12 ml of a boric acid solution (0.65 mol/l boric acid, adjusted to pH 4.35 with Na_2-EDTA). After washing the column with a further 8 ml water, normetanephrine, metanephrine and 3-methoxytyramine were eluted with 7.5 ml 4 mol/l ammonia.

The boric acid eluate (containing the catecholamines) was mixed with 180 µl perchloric acid solution (9 mol/l) and the ammonia eluate (containing the metanephrines) was mixed with 400 µl of a perchloric acid solution (9 mol/l). An aliquot (1.5 ml) of each eluate was applied to a column packed with Sephadex G 10. Sephadex G 10 columns (5 × 70 mm) were prepared in long (7 mm) *Pasteur* pipettes as described previously (14). Numerous columns (at least 80) can be handled in one run by using automated pipettes. Before use we washed the packed columns with 3.0 ml of 15 mmol/l ammonia and 3.0 ml of a 15 mmol/l formic acid solution (the formic acid solution contained 0.1 mmol/l Na_2-EDTA). After samples had passed through the columns, 2.0 ml of 15 mmol/l formic acid was added. The Sephadex eluate was then injected into the HPLC. If Sephadex eluates had to be preserved until the next day, they were stored at 4 °C. The Sephadex columns were stored in 15 mmol/l ammonia.

Acid hydrolysis of conjugated catecholamines

Centrifuged urine (0.5 ml) was adjusted to pH 1.0 with concentrated hydrochloric acid, and dihydroxybenzylamine (500 ng) was added as internal standard. This mixture was then heated for 20 min in a boiling water bath, chilled, and treated as described for the determination of free catecholamines.

Chromatography

We used a liquid chromatograph (Model 3500 B; Spectra-Physics, Inc., Berkeley, CA) equipped with a C18 reversed-phase column (Nucleosil 5 C18; Macherey-Nagel, Düren, F. R. G.), in conjunction with a rotating disc electrochemical detector (13). Samples were injected with an Autosampler (Kipp, Delft, The Netherlands) equipped with a 100 µl loop. The potential setting of the electrode was 500 mV (for the catecholamines) and 750 mV (for normetanephrine, metanephrine and 3-methoxytyramine). For both assays the mobile phase consisted of 0.1 mol/l acetic acid adjusted to pH 4.0 with solid sodium acetate, 0.8 mmol/l 1-heptanesulphonic acid, 0.01 mmol/l Na_2-EDTA, and 40−80 ml of methanol per litre. We filtered the

solutions (0.5 µm pore size Millipore filter) before assay. Concentrations in urine samples were calculated with the aid of calibration curves obtained after the injection of pure standards. The peak height of the various compounds is linear over a range of at least 0.1 – 100 ng per injection.

Results

The determination of free and total catecholamines

In an earlier study (13) we concluded that a two step purification procedure is necessary for a reliable analysis of free catecholamines in urine. Purification of urine samples on Bio Rad prepacked ion-exchange columns, followed by isolation of the compounds on small Sephadex G 10 columns, resulted in excellent chromatograms (fig. 1).

Hydrolysis of urine samples increases dramatically the number of compounds interfering with the analysis. Figure 2 shows a typical chromatogram of hydrolysed urine. When the methanol concentration of the mobile phase was reduced to 40 ml/l the unknown peaks did not interfere with a reliable quantitation of the catecholamines. A 20 min hydrolysis period was found to be optimal (data not shown).

The analytical recoveries (mean ± SD) of 500 ng dihydroxybenzylamine added to urine samples and processed through the Bio Rad columns as well as the Sephadex G 10 columns, were: 87.3 ± 6.8% (n = 39). When the urine samples were hydrolysed a recovery for dihydroxybenzylamine of 84.5 ± 6.7% (n = 39) was found. Mean (± SD; n = 4) 24 h values, corrected for the recoveries of the internal standard, of the 4 volunteers were (in nmol),

noradrenaline: 198 ± 76;
adrenaline: 70 ± 61;
dopamine: 1267 ± 192;
conjugated noradrenaline: 724 ± 236;
conjugated adrenaline: 104 ± 38;
conjugated dopamine: 6448 ± 3478.

These data are in good agreement with the literature (15).
Individual 24 h values for the free and conjugated catecholamines are given in table 1.

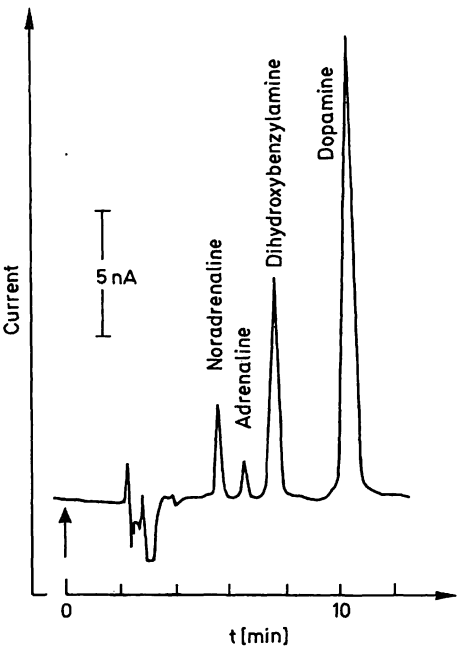

Fig. 1. Typical chromatogram of free catecholamines in a urine sample purified on a Bio-Rad ion-exchange column, then on a Sephadex G 10 column. Flow rate 1.0 ml/min; mobile phase: sodium acetate/acetic acid 0.1 mol/l, pH 4.0; 0.8 mmol/l 1-heptanesulphonic acid, and methanol, 70 ml/l; detector potential 500 mV.

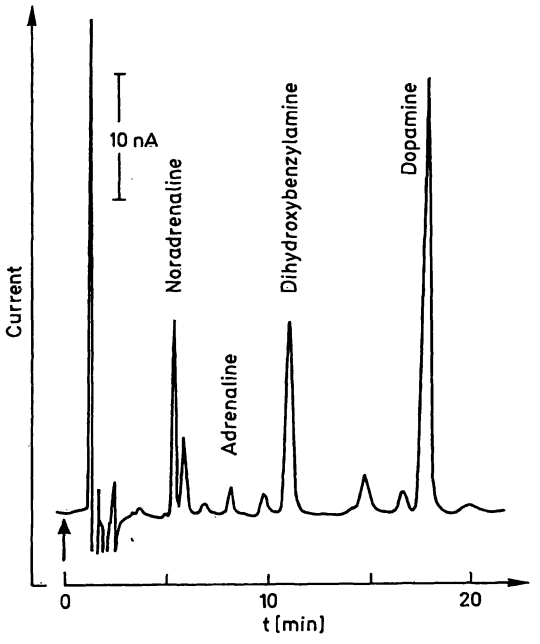

Fig. 2. Typical chromatogram of total catecholamines in an acid-hydrolysed urine sample purified on a Bio-Rad ion exchange column, then on a Sephadex G 10 column. Chromatographic conditions as in fig. 1 except that the methanol content was 40 ml/l.

Tab. 1. Individual 24 h values (nmol) of free, conjugated and methylated catecholamines of the 4 volunteer subjects.

Subject	Adrenaline		Metanephrine	Noradrenaline		Normetanephrine	Dopamine		3-Methoxytyramine
	free	conjugated		free	conjugated		free	conjugated	
1	162	217	138	176	527	106	1162	2468	134
2	41	49	126	307	1017	114	1506	5130	238
3	38	81	88	177	814	71	1331	10629	204
4	38	72	151	129	538	88	1071	7565	181

The determination of normetanephrine, metanephrine and 3-methoxytyramine

With a mobile phase similar to that used for the assay of the catecholamines, normetanephrine, metanephrine and 3-methoxytyramine were reliably quantified. A typical chromatogram is shown in figure 3. The potential setting of the electrode was 750 mV. As no appropriate internal standard was available at the time of this investigation, we added external standards to a number of urine samples. The recoveries (± SD) of 500 ng external standards were (n = 13 each),

normetanephrine: 93.5 ± 5.4%;
metanephrine: 82.7 ± 5.4%, and
3-methoxytyramine: 93.8 ± 9.0%.

Mean (± SD) 24 h values, corrected for the recoveries of the external standards, in urine obtained of the 4 volunteers were,

normetanephrine: 95 ± 19 nmol;
metanephrine: 125 ± 27 nmol; and
3-methoxytyramine: 189 ± 43 nmol.

These values are in the same range of data that have appeared in the literature (10, 11). Individual values are given in table 1. It is our experience that acid as well as enzymatic hydrolysis increases the number of electrochemically detectable compounds in biological samples. Hydrolysed samples could not be qualified because of poor resolution of the chromatograms.

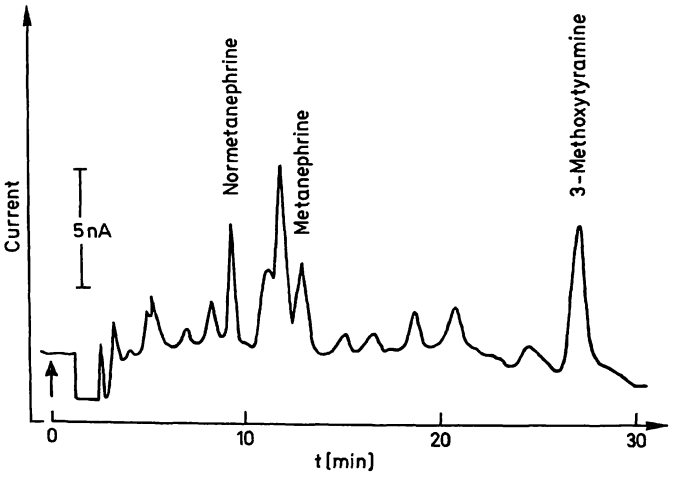

Fig. 3. Typical chromatogram of free metanephrines in a urine sample purified on a Bio-Rad ion-exchange column, then on a Sephadex G 10 column. Chromatographic conditions as in fig. 1, except that the detector potential was 750 mV.

The repeated use of the columns

Sephadex G 10 columns do not require regeneration. The columns can be used hundreds of times during at least 6 months without decreased recovery. Optimal recoveries of the analytes are obtained when the columns are at least one week old (14). The Sephadex G 10 columns can also be used for purification of homovanillic acid and 5-hydroxyindoleacetic acid in urine samples (13). The Bio Rad prepacked columns were regenerated as follows: after elution of the catecholamines or metanephrines, the columns were washed successively with 0.02 mol/l ammonia (10 ml), water (2 times 10 ml), then resuspended in 0.1 mol/l phosphate-citrate buffer pH 5.5 (2 times; only when metanephrines are not to be assayed), water (1 time 10 ml), and 1 g/l Na_2-EDTA of pH 6.5 (3 times 10 ml). Columns are stored in the EDTA solution; when used again the pH of the EDTA solution should be 6.5 ± 0.1. When regenerated in this way, the Bio Rad columns can be used for at least 50 times.

The circadian rhythms of free, conjugated and methylated catecholamines in urine

The 24 h excretion patterns of the various compounds determined for 4 volunteers are shown in figures 4–7. Values were expressed as µmol/mmol creatinine. A clear circadian rhythm was observed in the case of free adrenaline (fig. 4) and free noradrenaline (fig. 5) but not for free dopamine (fig. 6). In contrast to the free catecholamines, we found no clear rhythm in the case of the conjugated compounds. There is an increase in the variation of the data when conjugated catecholamines are compared with free catecholamines. A pronounced increase of conjugated dopamine during the evening was apparent in the urine of two of the four bus drivers.

As in the case of conjugated catecholamines, we found no circadian effects in the patterns of free normetanephrine, metanephrine and 3-methoxytyramine (fig. 7).

Discussion

In previous reports we emphasized (13, 14) the usefulness of a small Sephadex G 10 column in combination with a disposable Bio Rad ion exchange column for the rapid purification of catecholamines and related metabolites from biological samples. The present study shows that this procedure is also useful for purification of hydrolysed catecholamines as well as for free normetanephrine, metanephrine and 3-methoxytyramine.

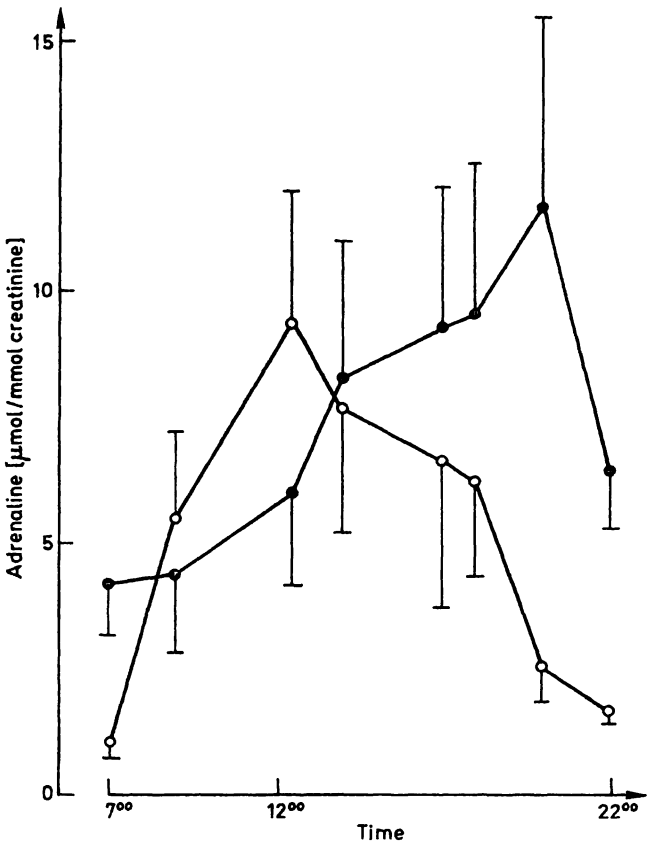

Fig. 4. The 24 h excretion pattern of free (o—o) and conjugated (●—●) adrenaline for 4 volunteers. Values are given in µmol/mmol creatinine ± S. E. M.

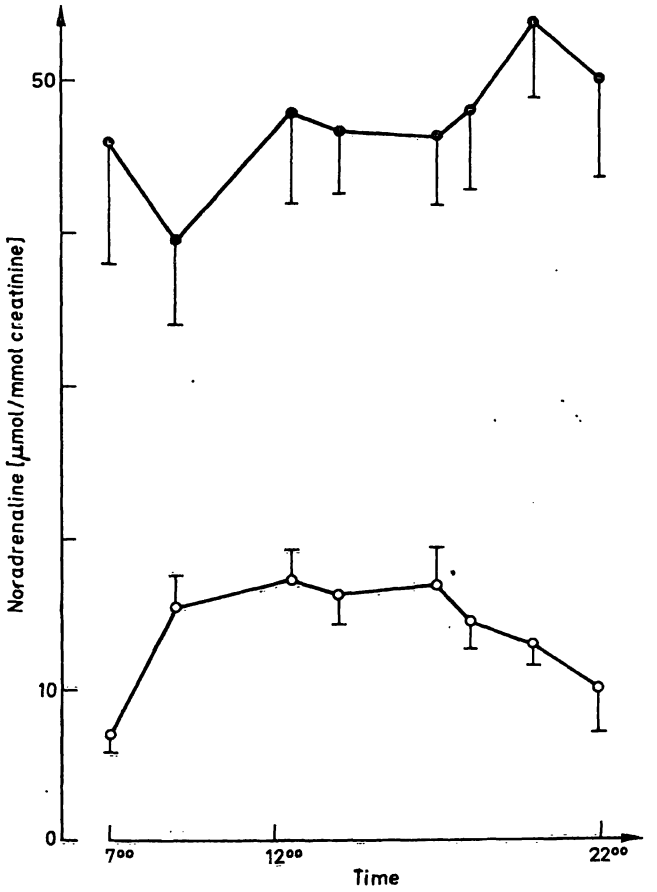

Fig. 5. The 24 h excretion pattern of free (o—o) and conjugated (●—●) noradrenaline for 4 volunteers. Values are given in µmol/mmol creatinine ± S. E. M.

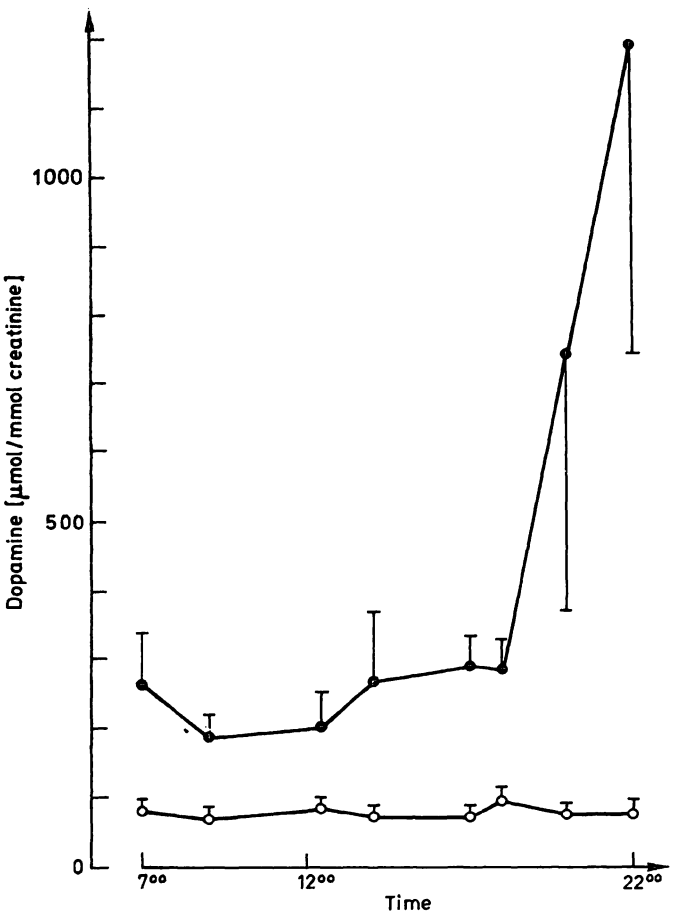

Fig. 6. The 24 h excretion pattern of free (o—o) and conjugated (●—●) dopamine for 4 volunteers. Values are given in µmol/mmol creatinine ± S. E. M.

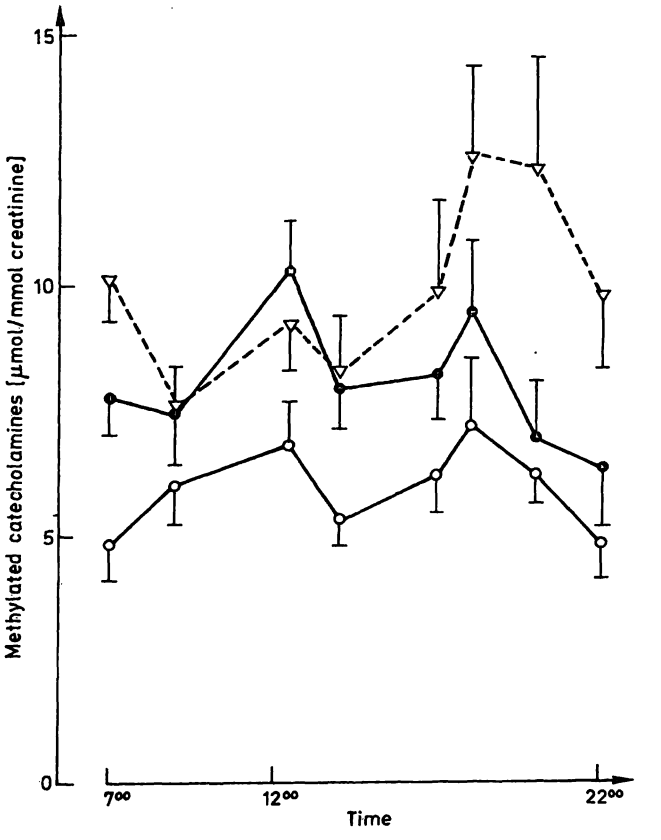

Fig. 7. The 24 h excretion pattern of free metanephrines for 4 volunteers. Values are given in µmol/mmol creatinine ± S. E. M.

●—● Metanephrine
o—o normetanephrine
▽---▽ 3-methoxytyramine

The Sephadex G 10 and the Bio-Rad column procedures are both easy to perform and suitable for routine analysis of large numbers of samples. During the purification steps the urine samples are diluted several times, but the amount of the present free and methylated catecholamines is sufficiently high to allow quantitation by HPLC with electrochemical detection.

The simultaneous approach, the high recoveries (84–94%) and the good precision (5–9%), means that the method is of potential use for application in psychoendocrine research and clinical chemistry as well. The possibility of reuse of both types of columns is a great advantage for routine procedures. In our laboratory 30 samples are processed in a working day.

Various research groups have reported that the urinary concentrations of free adrenaline as well as free noradrenaline display a circadian rhythm (12, 16). Although the majority of the catecholamines in urine is present as conjugates, these compounds have received little attention in this respect. We therefore investigated the relationship between free and conjugated catecholamines. Our data show that conjugated catecholamines behave differently when compared with the corresponding free amines. The circadian rhythm is lost and much more variation is present in the values. This finding is in agreement with the assumption that much of the conjugated catecholamines in blood and urine is derived from the diet (17). An efficient sulphoconjugating enzyme barrier localized in the intestine probably protects the body against the catecholamines present in the food (17). The increase of conjugated dopamine during the evening was observed in the urine of two of the four bus drivers; this increase can be explained by consumption of catecholamine-rich fruit such as bananas or oranges (18). Our findings are in agreement with a recent study of *Linnoila* et al. (19), who concluded that estimation of the 24 h values of total catecholamines is not worthwhile because of the large intraindividual variability of these values. However, it should be pointed out that in contrast to the total catecholamines, the 24 h values for the 3 free catecholamines display only a very small intraindividual variation (manuscript in preparation). Free urinary levels of the catecholamines are probably not influenced by exogeneous sources present in the diet; their values reflect therefore an interesting individual parameter of which the significance is hardly understood. Future studies should reveal which processes (such as transmitter release, metabolism or renal handling) contribute to the final concentrations of the free catecholamins in urine.

In studies on the relation between sympathoadrenal activity and transmitter function, the free methylated metabolites of the catecholamines were ignored. Recently *Kopin* et al. (5) have emphasized the importance of methylated catecholamines for evaluation of transmitter function. In this view it is believed that normetanephrine is formed from active noradrenaline outside the sympathetic neuron. The extraneuronal localization of catechol-O-methyl transferase is in accord with this assumption. We therefore investigated the relationship between catecholamines and corresponding methylated metabolites. Because of the involvement of dietary influences in the conjugation of catecholamines we were primarily interested in the concentration of non-conjugated normetanephrine, metanephrine and 3-methoxytyramine.

In the 24 h patterns we could not detect similarities between the patterns of free catecholamines and their corresponding (non-conjugated) methylated metabolites. The high adrenaline levels excreted by subject A were not reflected in the urinary metanephrine concentrations; moreover the circadian rhythms in noradrenaline and adrenaline were not present in the case of normetanephrine and metanephrine. Methylation of catecholamines seems not to be directly related to the functional release of these compounds.

Conclusion

We conclude that the most significant information about the transmitter function of catecholamines is obtained from determination of the free compounds; in this context, values for the conjugated and methylated metabolites do not seem to add much extra information.

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